details have emerged regarding the mechanism of GPCR-Gαβγ complex formation during signaling initiation because of the intrinsic instability of such complexes both *in vivo* and *in vitro* in the presence of detergent micelles. To overcome this limitation, we examined the utility of a range of membrane mimetics for stabilizing GPCR-Gαβγ complexes using the model system pair rhodopsin-transducin responsible for visual transduction. This system was chosen for its spectroscopic properties that can be easily followed as a measure of complex stability. Our results support the dependence of GPCR-Gαβγ complex formation on membrane morphology and nonspecific electrostatic interactions between membrane components and membrane protein binding molecules.

159-Plat

Quantitative FRAP Analysis Demonstrates that Raft Protein Clustering Alters N-Ras Depalmitoylation, Membrane Interactions and Activation Pattern

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The trafficking, membrane localization and lipid raft association of oncogenic Ras proteins dictate their isoform-specific biological responses. Accordingly, their spatiotemporal dynamics are tightly regulated. While extensively studied for H- and K-Ras, such information on N-Ras, an etiological oncogenic factor, is limited. Here, we report a novel mechanism regulating the activation-dependent spatiotemporal organization of N-Ras, its modulation by biologically-relevant stimuli, and isoform-specific effects on signaling. We applied patch/FRAP, FRAP beam-size analysis and a novel quantitative FRAP analysis of the diffusion and membrane binding kinetics of non-integral membrane proteins to investigate N-Ras membrane interactions. Clustering of raft-associated proteins, either glycosylphosphatidylinositol-anchored influenza hemagglutinin (HA-GPI) or fibronectin receptors, selectively enhanced the plasma membrane-cytoplasm exchange of N-Ras-GTP (preferentially associated with raft domains) in a cholesterol-dependent manner. EM analysis showed N-Ras-GTP localization in cholesterol-sensitive clusters, from which it preferentially detached upon HA-GPI crosslinking. HA-GPI clustering enhanced the Golgi-accumulation and signaling of EGF-stimulated N-Ras-GTP. Notably, the crosslinking-mediated enhancement of N-Ras-GTP exchange and Golgi accumulation strictly depended on depalmitoylation. We propose that the N-Ras activation pattern (e.g., by EGF) is altered by raft protein clustering, which enhances N-Ras-GTP raft localization and depalmitoylation, entailing its exchange and Golgi accumulation following repalmitoylation. This mechanism demonstrates a functional signaling role for the activation-dependent differential association of Ras isoforms with raft nanodomains.

160-Plat

Impact of Charcot-Marie-Tooth Type 2B Disease-Associated Rab7 Mutations on Signaling and Axonal Trafficking of NGF/TrkA

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Charcot-Marie-Tooth type 2B (CMT-2B) is a neurodegenerative disease characterized by terminal axonal death. Genetic analysis from human CMT-2B patients revealed four missense point mutations (L129F, K157N, N161T, V162M) in their genes encoding a small GTPase Rab7, a marker for late endosomes in the degradation pathway. The exact mechanism of how Rab7 mutants cause CMT-2B remains poorly understood. Here, we analyzed the effect of Rab7 mutants on the signaling and axonal transport of a nerve growth factor (NGF) receptor - TrkA. Fluorescent protein-engineered Rab7 and TrkA were transfected in rat embryonic dorsal root ganglia neuronal cells. Axonal transport of Rab7- and TrkA-containing endosomes was followed by time-stamped live cell fluorescence microscopy. We found that TrkA moves roughly twice as fast as Rab7s, among which CMT-2B associated Rab7 mutants outpace wt-Rab7. Curiously, endosomes co-transfected with both Rab7 and TrkA move even slower than those with singly transfected Rab7. Western blot analysis from Rab7/TrkA-cotransfected PC12 cells showed that the level of phosphorylated TrkA is lower in Rab7 mutants that that in wt-Rab7. Our results suggested that Rab7 mutants can potentially contribute to CMT-2B by dis-regulating NGF-TrkA signaling via perturbing the balance between retrograde and anterograde axonal transport processes. These results imply that axonal transport can be a potential treatment target for CMT-2B neurodegeneration.

161-Plat

Lipid Membrane Association of the T Cell Antigen Receptor ζ Subunit: Affinities and Structure

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The T cell antigen receptor (TCR) is a cell surface protein complex that binds peptide antigen fragments presented by the Major Histocompatibility Complex (MHC). The interaction of TCR with MHC leads to T cell activation and initiation of an immune response,[1] but the molecular details of the transmembrane signaling process remain unknown. TCRζ is the 115 amino acid long cytosolic signaling domain of the CD3-T cell receptor complex that plays a central role in this process. TCRζ is predominantly unstructured[2] and carries several copies of the immunoreceptor tyrosine-based activation motif (ITAM). ITAMs become phosphorylated upon receptor engagement, constituting an early and obligatory event in the signaling cascade that blocks lipid association and lipid-associated conformational rearrangements[3] of TCRζ. We present a characterization of the affinity of TCRζ to tethered lipid bilayers by SPR and a structural study of its association with such membranes using neutron reflectometry (NR). We studied the binding of unphosphorylated TCRζ to highly charged stBLMs rich in anionic phosphatidylserine (PS) or phosphatidylglycerol (PG) and observed affinity constants of $K_d \approx 10$ μM. Minute amounts of phosphoinositides ($PI(4,5)P_2$) increase the membrane affinity of the protein by more than 10-fold. An NR characterization of TCRζ in the membrane-associated state shows the major portion of the protein interfacially associated with the bilayer surface and a minor protein penetrating the bilayer deeply. This is consistent with a model in which an α-helical structure is aligned on the membrane surface that may be connected via a hinge with another α-helical segment inserted into the bilayer.

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162-Plat

Membrane Protein Cluster Titration in Early Lymphocyte Signaling

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T cells are known to be sensitive to extremely small numbers of agonist peptides. Early T cell signaling originates in and is sustained by T Cell Receptor (TCR) microclusters. Recent reports indicate that T cell triggering thresholds are determined by the number of agonist peptide in individual TCR microclusters, a conclusion supported by the observation that 2D peptide-MHC kinetics are characterized by unexpectedly fast on-rates. Yet, questions remain regarding the interplay, if any, between signaling microclusters associated with the plasma membrane. We have studied the relationship between peptide number and protein cluster formation in early T cell signaling by direct, simultaneous observation of fluorescently labeled agonist peptides and the early T cell signaling molecule Zap70 genetically fused to EGFP in primary murine T cells. Our experiments replace the antigen-presenting cell with a supported lipid bilayer (SLB) chemically functionalized with peptide-MHC and ICAM-1. This approach allows us to titrate the density of triggering agonist peptide, while also providing a flat interface suitable for high-resolution TIRF imaging. By integrating micro-patterned chromium supports onto a cover glass we can also segregate the SLB into an array of spatially distinct micron-scale reaction centers. Using this method we measure antigen input vs. TCR triggering output in living primary T cells. These results have important implications concerning the role of cooperativity between adjacent TCR clusters in tuning the sensitivity of T cell triggering by exogenous agonist peptides.

Platform: Membrane Dynamics & Bilayer Probes

163-Plat

Nanoscale Interactions of Lipids and Proteins in Live Cell Membranes Revealed by STED Nanoscopy

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The plasma membrane seems to be dynamically structured in regions of different composition and function. However, the spatial and temporal scale

of the respective membrane structure is not directly accessible by the diffraction limited resolution of conventional far-field optical microscopes. We report the detection of the membrane heterogeneities in nanosized areas in the plasma membrane of living cells using the superior spatial resolution of stimulated emission depletion (STED) far-field nanoscopy. By combining a (tunable) resolution of down to 30 nm with tools such as fluorescence correlation spectroscopy (FCS), we obtain new details of molecular membrane dynamics. Sphingolipids are transiently (~ 10 ms) trapped on the nanoscale in cholesterol-mediated molecular complexes, while glycerophospho-lipids diffuse freely. The results are compared to STED experiments on model membranes, which highlight that the nanoscale trapping in cells is not correlated with liquid order partitioning in model systems. The novel observations shine new light on the distribution and interaction of lipids and proteins in the plasma membrane with respect to the lipid raft hypothesis.

164-Plat
Multivalent Chelator Lipids for Targeting and Manipulation of Proteins in Membrane Nanodomains
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Membrane nanodomains based on phase-segregation of lipids have emerged as a key organizing principle of the plasma membrane. They have been shown to play important roles in signal transduction and membrane trafficking. We have developed lipid-like probes carrying multivalent nitrilotriacetic acid (tris-NTA) head groups for selective targeting of His-tagged proteins into liquid ordered or liquid disordered lipid phases. The stable, non-covalent interaction of His-tagged proteins to the tris-NTA moiety can be employed not only for efficient specific tethering of spectroscopic probes, but also for versatile manipulation of membrane nanodomains. In giant unilamellar vesicles strong partitioning of tris-NTA lipids into different lipid phases was observed. For a saturated tris-NTA lipid, at least 10-fold preference for the liquid ordered phase was found. In contrast, an unsaturated NTA lipid shows a comparable preference for the liquid disordered phase. Simular partitioning of the tris-NTA lipids was observed in solid-supported membranes on mica. Partitioning into submicroscopic membrane domains formed in solid-supported membranes was confirmed by superresolution imaging techniques (FPALM, STED). Single molecule tracking of His-tagged proteins tethered to solid-supported phase-separating membranes revealed clear differences in the diffusion behavior of the different NTA-lipids. By using vesicles as a carrier, multivalent NTA lipids were efficiently incorporated into the plasma membrane of live cells. After formation of giant plasma membrane vesicles (GPMV), efficient partitioning of the lipid probes into the respective membrane phases was confirmed. We have employed these probes for exploring lipid diffusion, morphology and spatiotemporal dynamics of membrane nanodomains in *vitro* and live cells by single molecule tracking and STED FCS.

165-Plat
Model of Self Reproducing Vesicles
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Evolution of molecular assemblies toward a cellular life in prebiotic world is fascinating research filed in soft matter science. So far extensive investigations have been performed to construct molecular model system which expresses similar dynamics as those of a life system using well-defined organic molecules. Especially modeling of a self-reproducing vesicular system is a key concept to understand origin of the cellular life.
In typical self-reproducing vesicle systems, membrane precursors are converted into membrane molecules with the help of a catalyst and the membrane molecules induce the formation of daughter vesicles. The self-reproducing vesicle systems have two pathways for the topology transition, the birthing and the budding. In the birthing pathway, new synthesized membrane molecules form daughter vesicles inside a mother vesicle. When the daughter vesicle grows to a certain size, it extrude through the membrane of the mother vesicle to the environment. On the other hand, in the budding pathway, the mother vesicle deforms to pear-like shape and is divided into two independent vesicles.
We established a model self-reproducing vesicle system without the membrane molecule synthesis route. The model vesicle composed of cylinder- and inverse-cone-shaped lipids formed inclusion vesicles inside the mother vesicle and the inclusion vesicles were then expelled by a temperature cycling. By changing the vesicle composition, the mother vesicles showed the budding type self-reproduction pathway. A key concept of this system is the coupling of the main-chain transition and the shape of lipids.

166-Plat
Lipid Membrane Deformation in Response to a Local pH Modification
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During cell life, membranes are subjected to a variable and inhomogeneous environment. These local variations can be strongly related to biological processes. For instance, in the inner membrane of mitochondria, ATP synthesis is tightly coupled to the local pH. It is therefore interesting to study the response of a lipid membrane to a local modification of its environment.
In our previous works (Khalifat et al., Biophys. J., 2008; Fournier et al., PRL, 2009; Bitbol et al., J. Phys.: Condens. Matter, 2011), we designed an experiment where the local pH at an artificial membrane (lipid giant unilamellar vesicle) is modified using microinjection. We showed that modifying the local pH induced a dynamic membrane deformation. We also developed a theoretical description of the dynamics of a membrane subjected to a local modification affecting its physical properties. It involved elaborating a local version of the area-difference elasticity model and accounting for the friction between the two monolayers of the bilayer membrane.
We have now extended our theoretical model to account for the diffusion of the membrane-modifying reagent in the surrounding solution. We solved numerically the equations describing the dynamics of the membrane in this case. We showed that the effect of diffusion from the local reagent source is generally important, but that there are experimentally accessible cases where the dynamics is dominated by processes intrinsic to the membrane, such as the relative sliding of its two monolayers. We compared the predictions of our extended theoretical model to experimental results regarding the dynamics of lipid (PC/PS-) membrane deformation during and just after the local delivery of a basic solution. Moreover, we used a pH-sensitive fluorescent membrane marker to have a direct experimental visualization of the membrane pH profile together with the deformation.

167-Plat
The Investigation of Lipid Membrane Deformation in Giant Unilamellar Vesicles using Microfluidic Technology
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ETH Zurich, Zurich, Switzerland.
Membrane curvature is known to play an important role in cell growth, division and movement. Different lipid and membrane proteins can influence the cell's shape by changing the membrane curvature. Developing new technologies is vital for investigating and understanding the processes involved when membranes deform. We present an approach to study membrane curvature in giant unilamellar vesicles (GUVs) using microfluidic technology. Micron-sized channel networks in microfluidic devices are ideally suited for the manipulation and imaging of biological structures. The ability to confine liposomes in highly controlled environments allows them to be exposed to precise mechanical stresses for membrane analysis. The device presented here was fabricated in polydimethylsiloxane (PDMS) and consists of microchannels where GUVs can be introduced or grown within using the electro-swelling method. They can be immobilised at specific positions using a biotin-PEG-cholesterol linker patterned on to the glass bottom of the channel, then subjected to mechanical forces using an actuated PDMS membrane the above the channel. Initial experiments show that the GUVs are stable when subjected to a force from the PDMS micro-stamp allowing them to be imaged. To obtain high resolution 3-D images of the GUVs as they are deformed, we have combined established imaging methods with integrated optical components. More precisely, a silver coated micro-mirror inside the PDMS wall allows objects within the channels to be imaged from the side as well as from below. This enables high-resolution 3-D imaging in the axial plane using a confocal microscope. With the aim to study the effects of curvature on phase separation, we have created lipid domains in GUVs using a ternary lipid mixture of SM/DOPC/Cholesterol and DLPC/DPPC/Cholesterol. The device has the potential for investigating the mechanosensitivity of membrane bound proteins and whole cells.